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Surface dynamics of GluN2B-NMDA receptors controls plasticity of maturing glutamate synapses

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Editor: Andrea Leibfried

1st Editorial Decision 04 September 2013

Thank you for submitting your manuscript to The EMBO Journal. Your study has now been seen by two referees and their comments are provided below. Dr. Andrea Leibfried is the handling editor for your manuscript, but as she is away on vacation at the moment I am stepping in as secondary editor on the paper.

As you can see, both referees find the analysis interesting and insightful. However, they also find some parts of the paper too preliminary and that further analysis would be needed to strengthen the findings reported. The concerns raised are clearly outlined below. Should you be able to address the concerns raised in full, then we would like to invite you to submit a suitably revised manuscript for our consideration. I should add that it is EMBO Journal policy to allow only a single round of revision, and that it is therefore important to address the raised concerns at this stage.

Thank you for the opportunity to consider your work for publication.

REFEREE COMMENTS

Referee #1

The authors use cutting edge technology to demonstrate that lateral diffusion of postsynaptic NMDARs with GluN2B subunits increases upon glycine-induced LTP (cLTP). Intriguingly, application of antibodies against the extracellular N-termini of GluN1 and GluN2B prevents the increase in lateral diffusion of GluN2B and the induction of cLTP in cultured neurons and of electrically induced LTP in acute hippocampal slices. The inhibition of increased diffusion and of cLTP was also true for autoantibodies against the GluN1 N-terminus from patients with autoimmune encephalitis. Because the antibodies cross-link NMDARs the authors conclude that these effects are due to immobilization of GLuN2B-containing NMDARs. Importantly, this cross-linking did not affect localization of NMDAR under basal conditions nor NMDAR-mediated currents. A further remarkable finding is that cLTP-induced accumulation of CaMKII, which is mediated by GluN2B binding, is prevented by cross-linking antibodies.

Major Concerns

1. The prevention of cLTP-induced postsynaptic CaMKII accumulation by the cross-linking antibodies provides a second potential explanation to how those antibodies prevent LTP: rather than preventing the increase in lateral diffusion they might induce a conformational change in the cytosolic site of the NMDAR that prevents CaMKII binding, which in turn impairs LTP. It might not be possible to differentiate between those two possibilities at this point and the observed effects are clearly of broad interest. However, the authors might want to try and find another way of crosslinking GluN2B containing NMDARs (use an external tag that allows cross-linking independent of antibodies or engineering cysteine residues for disulfide bond formation with a membrane impermeant oxidant) to prove the importance of lateral diffusion of GluN2B. Whether or not the authors can find a method to solve this question (which is not a trivial task at this point), they should address whether the cross-linking antibodies affect CaMKII binding; for instance, they could test whether the antibodies impair coimmunoprecipitation of CaMKII with GluN2B under basal conditions or after cLTP.

2. In Fig. 1 the authors show that KN62 and KN93, both of which inhibit various CaM-dependent kinases and some other targets, prevent the increase in the mobility of the NMDAR subunit GluN2B upon induction of chemLTP with glycine. It would be important to confirm the role of CaMKII with a more specific CaMKII antagonist (e.g., membrane-permeant AIP). The same is true for the inhibition of CK2 with TBB and mGLuR5 with MPEP-again for both drug targets a second drug or potentially knock down should be used.

Minor Concerns

1. The claim that Sanz-Clemente et al. 2010 shows that CK2 is important for LTP (last line on p. 7) seems not correct to me-this paper mainly shows that CK2 is important for GluN2B removal from synapse (which can, however, affect LTP).

2. Fig. 5D needs better explanation of what is actually shown.

3. The authors state in Discussion on p. 17 'GluN2B surface dynamics required for LTP was prevented by AP5 but was independent of NMDAR synaptic current or calcium influx.' This statement implies to me that the glycine-induced change in GluN2B diffusion did not require calcium influx. I believe the authors did not address this point (other than using AP5) and need to modify this statement (e.g., 'was independent of ALTERATIONS in NMDAR synaptic current or calcium influx' if that is what the authors intended to say as those parameters were unchanged).

Referee #2

NMDA receptors are known to play a key role in regulating plasticity at excitatory synapses. In this paper, the authors propose that GluN2B subunit surface dynamics are critical to controlling synaptic plasticity in developing hippocampal neurons. Using a collection of techniques, ranging from high resolution single nanoparticle imaging, ensemble imaging techniques (FRAP) to electrophysiology, they observe that GluN2B-NMDAR rapidly exit the synapse upon a chem. LTP stimulus and that this process is NMDAR-, mGluR5, casein kinase II-, and CAMKII- dependent. Preventing this surface redistribution, through antibody cross-linking, alters activity-induced changes in CAMKII intracellular dynamics and ultimately blocks LTP.

This is a potentially important and interesting study further supporting a critical role for NMDAR trafficking in excitatory plasticity. However some aspects of the manuscript seem a little preliminary and would need to be improved. A particular concern is the lack of any major mechanistic advance. The idea that alterations of excitatory synapse strength are accompanied by rapid changes in ionotropic glutamate receptor lateral mobility has already been pioneered by the authors and others. The important new insights are that lateral mobility specifically of NMDAR2B subunits may underpin this plasticity and impact on CamKII localisation. However the mechanistic description underlying how NR2B Diff. increase occurs or exactly how this contributes to altered CamKII trafficking or LTP is poorly described. A concern is that a majority of the experiments are dependent on altering receptor lateral mobility using only 1 approach - namely antibody cross-linking. While the authors do perform several controls for this interference the manuscript would be much more compelling if alternative molecular interventions (based on clearer mechanistic insight) had also been used. For example if mutants of the NR2B subunit which block the increase in NR2B lateral mobility or cross-talk with CamKII stabilisation / trafficking were also shown to block LTP.

Major Points:

1) in figure 1 the results of the diffusion dynamics experiments using quantum dots suffer from a lack of clarity. It's a little unclear whether the scores presented relate to synaptic, extra-synaptic or total trajectories. I think the results of the synaptic diffusions scored are provided but a little more clarification would improve this section. In any case, extended Mean Square Displacement (MSD) curves or calculations of synaptic dwell times (which is mentioned in the supplementary methods but doesn't appear in any other section) might serve to illustrate these results more clearly i.e. highlight constrained diffusion at synapses versus brownian motion extrasynaptically. Panel E is not adequately described in the accompanying text. Do the symbols represent the inter quartile range (IQR) of diffusion scores for each condition? In panel F, does the "difference change post chemLTP (%)" refer to changes in the median score across conditions? If mean scores are provided, then this would be in contrast to most of the SPT field, where medians scores are provided since diffusion scores can vary across orders of magnitude.

2) The experiments using kinase blockers are very preliminary. There is no clear mechanistic understanding of the contribution played by these kinase pathways to the process. Is this dependent on direct NR subunit phosphorylation or an indirect effect? Why do two entirely different kinase signalling pathways have exactly the same effect in terms of completely blocking the diff change. In there current form these kinase blocking experiments add very little to the overall manuscript and simply serve to highlight the lack of any clear mechanism.

3) I am a little concerned regarding whether or not the 'diff. plasticity' is really developmentally regulated. The premise for this is based on the observation that the NR2B diff change is lost in older cultures while potentiation remains. But the read out for this is a preserved 11% 'potentiation' of an exogenous SEP GluR1 subunit. I don't think strong conclusions can be drawn from this. It's equally likely that the older cultures are simply harder to chem. LTP potentiate and that is why there is no increade in NR2B Diff.

4) While I understand the premise of using SPT to establish a ratio of synpatic versus perisynaptic co-localisation in Fig.2 , I don't fully understand how the data was quantified. The text refers to "a 500-frames stack was used to elaborate the distribution map of either GluN2A- and GluN2B-NMDAR". How was stack used exactly? Wouldn't the QDs move around in the 500 frame stack, leading to double counting? Would it be better to take a single image at a specified timepoint both pre and post chemLTP and compare the distribution of QDs relative to the synaptic zones. Some further clarification (in the methods or elsewhere) could prevent possible misunderstandings of the data.

5) The authors propose that the increase in NR2B lateral diff. contributes to a change in 2A/2B ratio. But how would a loss of receptors drive potentiation and what does this do to the amount of NR2A at the synapse? I thought the premise of the Bellone and Nicoll study was that this would 'free up' slots for NR2A at the synapse? Would the cross-linking not also block a movement of NR2A receptors into the synapse? How do the authors think this fits with previous work?

6) While the control experiments in Fig3 that prove that the x-linking process doesn't impair normal synaptic function are rigorous, they don't directly contribute to the impact of the paper, as this technique has been previously described (these papers are adequately referenced in the manuscript). Could some of Figures 3 and 4 be combined (as well as the corresponding sections within the Results), with some panels provided as supplementary figures.

7) In figure 5 as GluN2A experiences no surface redistribution following chemLTP (Figure 1), it could be argued that GluN2A x-linking would be insufficient to block LTP. It would be nice to actually see this hypothesis tested.

8) The data relating to the experiments performed using anti-NMDAR autoimmune antibodies from patients with cognitive dysfunctions don't add much to the story and seem a little preliminary. There is scant information regarding their provenance - is this pooled IgG from multiple patients? Also the mechanisms of how these antibodies act is unclear if the antibodies are mixed they may be doing several things For example are they targeting different epitopes of NR subunits? What impact do these antibodies have on NR function. It's quite possible some of the patient IgG effects are not due to cross-linking per se.

These data might be better suited to amore in depth study for a more clinically focused journal but if they are to be kept then their presentation might be improved. A quantification of the qd trajectories with patient and control IgG, represented in panel B, would be helpful and straightforward to create e.g. panel B of Figure 3. Are the lower panels of Figure 6 panel D comparable to panels C and D of Figure 5. I'm unsure why a.u. was employed as the scale of measurement in the lower left graph of Figure 6 panel D, when the corresponding graph in Figure 5C uses %. Also, with particular reference to Control IgG, the numbers in Figure 6 differ quite dramatically with the numbers in Figure 5C and 5D. In lower left panel of Figure 6D, the GluA1 syn. content almost doubles over basal, compared to a modest 10% increase observed in the same control experiment in Figure 5C. Likewise, a modest increase in potentiated synapses in the lower right panel of Figure 6D contrasts with an almost trebling in the corresponding experiment presented in Figure 5D. These discrepancies should be addressed.

9) The results on CamKII trafficking are potentially very interesting and could support an explanation for how NR2B Diff increase might underpin plasticity (in the absence of an increase in NR2A) but the data are mostly correlative and the underlying mechanism are not clear. Can the authors further address how an increase in NR2B Diff. alters CamKII trafficking and then demonstrate that blocking this blocks LTP.

Minor Points:

I find some of the text introducing quantum dots towards the beginning of the results section to be superfluous, as qds have been employed to study neuronal receptor dynamics for about 10 years and are relatively well established in the field. The results section might benefit from a more succinct treatment of this topic, especially given the sparsity of detail in the Methods sections, where this material might be more appropriately provided.

Page 14: "Because surface NMDAR also directly interact with PSD-95" requires adequate referencing.

Figure 1: For panel A, can a corresponding images of Homer fluorescence be provided.

Figure 2 Panel B & C: how representative are the GluN2A images of panel B, as they don't appear to correlate well with the results summarised in panel C? From panel B, the vast majority of receptors appear to be localised at the synapse, as opposed to the perisynpase, but the quantified data in panel C suggests the opposite is true (as the ratio of synaptic over perisynaptic is around 0.5).

Figure 2D: There could be some mismatch between the Figure and the time periods mentioned in the accompanying figure legend. It says the recording lasted 35mins (should it be 40mins?) and that fluorescence increased 35mins after chemLTP (should this be 30mins?).

Figure 2E: A control that allows for photobleaching, drift, etc, could be added to this graph, much like the first panel of Figure 1B.

1st Revision - authors' response 22 November 2013

Referee #1

1. …the authors might want to try and find another way of x-linking GluN2B containing NMDARs (use an external tag that allows cross-linking independent of antibodies or engineering cysteine residues for disulfide bond formation with a membrane impermeant oxidant) to prove the importance of lateral diffusion of GluN2B. Whether or not the authors can find a method to solve this question (which is not a trivial task at this point), they should address whether the cross-linking antibodies affect CaMKII binding; for instance, they could test whether the antibodies impair coimmunoprecipitation of CaMKII with GluN2B under basal conditions or after cLTP.

Response: We agree with the reviewer that engineering an alternative molecular strategy to crosslink NMDAR would constitute a nice additional asset to reinforce our conclusions. However, such approaches are not particularly easy to implement and the amount of work required to engineer another crosslink strategy would definitely not be manageable within the three months allowed for revision. We thank the referee for acknowledging the fact that this is experimentally challenging.
We however directly tackled the second proposal of referee, i.e. to perform co-immunoprecipitation experiments of CaMKII and GluN2B in control and x-link conditions. Indeed, LTP impairments observed in the presence of x-link could very well result from a reduced binding of CaMKII to GluN2B-NMDAR. We thus co-immunoprecipitated GluN2B subunits from hippocampal slices incubated either in control or x-link conditions and measured the amount of αCaMKII and phosphoCaMKII. As detailed in the revised manuscript (Figure 8A), GluN1-NMDAR x-linking, which prevents LTP, does not alter the binding between GluN2B and CaMKII, indicating that a x-linking-induced disruption of the GluN2B-CaMKII interaction is not responsible for the prevention of LTP. This important new piece of evidence is now discussed in the Results section of the revised manuscript (pages 15-16; Figure 8A).

In Fig. 1 the authors show that KN62 and KN93, both of which inhibit various CaM-dependent kinases and some other targets, prevent the increase in the mobility of the NMDAR subunit GluN2B upon induction of chemLTP with glycine. It would be important to confirm the role of CaMKII with a more specific CaMKII antagonist (e.g., membrane-permeant AIP). The same is true for the inhibition of CK2 with TBB and mGLuR5 with MPEP-again for both drug targets a second drug or potentially knock down should be used.

Response: In order to meet the referee's expectations, we have now performed additional series of experiments using other antagonists of CaMKII (AIP-2, KN93), CK2 (TMCB) and mGluR5 (MTEP) to test their impact on GluN2B-NMDAR dynamics during chemLTP. These additional data matched our initial observations since AIP-2, KN93, TMCB or MTEP prevented change in GluN2B dynamics during chemLTP, confirming that CaMKII, CKII and mGluR5 are involved in the surface redistribution of GluN2B-NMDAR associated with synaptic plasticity (Figure 7A-C). These additional results are now included in the Results section of the revised manuscript (pages 14-15, Figure 7A-C).

The claim that Sanz-Clemente et al. 2010 shows that CK2 is important for LTP (last line on p. 7) seems not correct to me-this paper mainly shows that CK2 is important for GluN2B removal from synapse (which can, however, affect LTP).

Response: Thank you for pointing this out. We fully agree and corrected the statement: "Thus, among the protein kinases listed above, CaMKII and CKII activities, which are required for GluN2B-NMDAR synaptic trafficking and LTP induction…" (page 14).

Fig. 5D needs better explanation of what is actually shown.

Response: We have now highlighted on the figure (now Figure 4) the position of glutamate synapses on spine, since these areas have been used to quantify the effects of chemLTP and various x-link procedures. These supplementary information are further indicated into figure legends.

The authors state in Discussion on p. 17 'GluN2B surface dynamics required for LTP was prevented by AP5 but was independent of NMDAR synaptic current or calcium influx.' This statement implies to me that the glycine-induced change in GluN2B diffusion did not require calcium influx. I believe the authors did not address this point (other than using AP5) and need to modify this statement (e.g., 'was independent of ALTERATIONS in NMDAR synaptic current or calcium influx' if that is what the authors intended to say as those parameters were unchanged).

Response: We agree and the statement was corrected accordingly (discussion section, page 19).

Referee #2

in figure 1 the results of the diffusion dynamics experiments using quantum dots suffer from a lack of clarity. It's a little unclear whether the scores presented relate to synaptic, extra-synaptic or total trajectories. I think the results of the synaptic diffusions scored are provided but a little more clarification would improve this section. In any case, extended Mean Square Displacement (MSD) curves or calculations of synaptic dwell times (which is mentioned in the supplementary methods but doesn't appear in any other section) might serve to illustrate these results more clearly i.e. highlight constrained diffusion at synapses versus brownian motion extrasynaptically. Panel E is not adequately described in the accompanying text. Do the symbols represent the inter quartile range (IQR) of diffusion scores for each condition? In panel F, does the "difference change post chemLTP (%)" refer to changes in the median score across conditions? If mean scores are provided, then this would be in contrast to most of the SPT field, where medians scores are provided since diffusion scores can vary across orders of magnitude.

Response: the results of the receptor dynamics imaging using quantum dots have now been clarified in this section. First, the localization of the receptor-QD complex has been better indicated when appropriate. Second, panel E have been better described in the accompanying text (figure legend). Third, the symbols represent the mean ± sem. Fourth, in panel F, the difference change after chemLTP represent the variation of the median of the instantaneous diffusion coefficient changes. This has been clarified. For notice, although we fully agree that the median is the correct expression measure for the diffusion data that are non-parametric, comparison between mean and median often provide similar outcomes. Fifth, although we fully agree with the referee that additional data, such as the dwell time, "might serve to illustrate the results", we did not include the dwell-time into the revised manuscript for the following reason. We observed that right after LTP induction a fraction of GluN2B-NMDAR diffuse away from synapse leaving a rather stable fraction in synapses. Consistently, when we measured the dwell-time of the synaptic GluN2B-NMDAR population during LTP we observed an "apparent" increase (before: 3.6 ± 0.3 s, n = 368 trajectories; during/after LTP: 4.3 ± 0.4 s, n = 163), consistent with the fact that remaining stable GluN2B-NMDAR were mostly contributing to this value. After careful considerations, it appeared quite challenging to measure the synaptic dwell-time of only the receptors that rapidly react to the LTP stimulus and run away from synapses. One could imagine performing a biased analysis of selected trajectories, based on diffusion parameters, which, we believe, has strong inherent limitation. Thus, at this stage, the diffusion coefficient perfectly reflects the dynamic and distribution change of GluN2B-NMDAR. Adding other dynamics parameters would not clarify the complex behaviors of all GluN2B-NMDAR. To reflect this point, we further emphasize in the revised manuscript that there is a remaining stable fraction of GluN2B-NMDAR even after LTP (page 21-22).

The experiments using kinase blockers are very preliminary. There is no clear mechanistic understanding of the contribution played by these kinase pathways to the process. Is this dependent on direct NR subunit phosphorylation or an indirect effect? Why do two entirely different kinase signalling pathways have exactly the same effect in terms of completely blocking the diff change. In there current form these kinase blocking experiments add very little to the overall manuscript and simply serve to highlight the lack of any clear mechanism.

Response: To address this concern we have now dug into the molecular mechanism of the activity-dependent interplay between GluN2B-NMDAR and CaMKII dynamics. We first found that in basal conditions, CaMKII regulates the surface dynamics of GluN2B-NMDAR through a phosphorylation-dependent mechanism since several CaMKII antagonists (KN62, KN93, AIP2) massively reduced the diffusion of GluN2B-NMDAR, both synaptically and extrasynaptically (Figure 7C). Interestingly, part of this regulation depends on the direct interaction between GluN2B and CaMKII since GluN2B-R1300Q/S1303D in which the C-terminal interaction site for CaMKII is prevented (Strack et al., J. Biol. Chem., 2000) also displays significantly reduced surface dynamics (Figure 7C). Moreover and quite strikingly, this mutant is not displayed away from synapses as wild-type GluN2B-NMDAR are during activity-induced potentiation, indicating that the direct interaction between GluN2B-NMDAR and CaMKII is required for this plasticity-elicited surface redistribution to occur (Figure 7D, E). Finally, we performed additional FRAP experiments showing that expressing this GluN2B mutant prevents chemLTP-elicited changes in CaMKII intracellular diffusion, suggesting that the direct interaction between GluN2B-NMDAR and CaMKII is also necessary for activity-induced changes in CaMKII intracellular dynamics to take place (Figure 8C). Therefore, we demonstrate that trough their cytosolic interaction, activity-induced changes in the surface diffusion of GluN2B-NMDAR also affect CaMKII intracellular dynamics in a crucial manner during synaptic plasticity induction. These results are now included in the Results section of the revised manuscript (Figures 7, 8, and corresponding text whole over the manuscript).

I am a little concerned regarding whether or not the 'diff. plasticity' is really developmentally regulated. The premise for this is based on the observation that the NR2B diff change is lost in older cultures while potentiation remains. But the read out for this is a preserved 11% 'potentiation' of an exogenous SEP GluR1 subunit. I don't think strong conclusions can be drawn from this. It's equally likely that the older cultures are simply harder to chem. LTP potentiate and that is why there is no increase in NR2B Diff.

Response: To tackle this point, we performed a new series of fEPSP electrophysiological recordings in adult hippocampal slices in which we tried to induce LTP in absence or presence of GluN1 x-link. We now demonstrate that if GluN1 x-link significantly reduced LTP in young hippocampi (Figure 5C, G), the same paradigm produces little, if any, effect on the induction of LTP in adult (Figure 5D). This new data set supports our claim that the process is developmentally-regulated, although we make it clear in the revised manuscript that we cannot conclude that NMDAR surface dynamics does not play any role in the adult (page 20).

While I understand the premise of using SPT to establish a ratio of synpatic versus perisynaptic co-localisation in Fig.2 , I don't fully understand how the data was quantified. The text refers to "a 500-frames stack was used to elaborate the distribution map of either GluN2A- and GluN2B-NMDAR". How was stack used exactly? Wouldn't the QDs move around in the 500 frame stack, leading to double counting? Would it be better to take a single image at a specified timepoint both pre and post chemLTP and compare the distribution of QDs relative to the synaptic zones. Some further clarification (in the methods or elsewhere) could prevent possible misunderstandings of the data.

Response: We apologize for the lack of clarity resulting from the apparent discrepancy between the representation of the successive locations of a single NMDAR/QD complex in Figure 2B and the way we calculated synaptic fractions in Figure 2C. Schematically, the way the analysis is performed to generate representations as in Figure 2B is rather simple. A 500 frame stack is obtained while tracking down a single NMDAR/QD complex. On each frame the receptor/particle complex is detected and precisely located, and so on for 500 frames. Then, those 500 locations are projected on a single image, providing the different high-resolution locations of this particular receptor/particle during this 500 frame stack, as depicted in Figure 2B.
Regarding synaptic fraction calculation (Figure 2C), hundreds of synaptic, perisynaptic and extrasynaptic receptor/particle complexes are simultaneously detected on each frame. Thus, we can then define a synaptic fraction for each frame by dividing the number of synaptic NMDAR/QD complexes by the total number of NMDAR/QD complexes detected at this given time point (which means that there is no double counting but 500 independent counting, one for each frame). The synaptic fraction for a dendritic field is then calculated as the mean of the synaptic fractions for each frame (Figure 2C). This has now been better explained in the Methods and supplementary methods in the revised manuscript.

The authors propose that the increase in NR2B lateral diff. contributes to a change in 2A/2B ratio. But how would a loss of receptors drive potentiation and what does this do to the amount of NR2A at the synapse? I thought the premise of the Bellone and Nicoll study was that this would 'free up' slots for NR2A at the synapse? Would the cross-linking not also block a movement of NR2A receptors into the synapse? How do the authors think this fits with previous work?

Response: Our data show that a fraction of GluN2B-NMDAR is displaced outside synapses during $_{chem}$LTP (first few minutes) whereas no change of GluN2A-NMDAR is observed during this time window. Consequently, the 2A/2B ratio is rapidly increased during the first few minutes of chemLTP protocol. In the Bellone and Nicoll study, they report a rapid (first few minutes) decrease contribution of GluN2B-NMDAR to synaptic transmission, fully consistent with our imaging data. In addition, they report that 5 min (and later) after LTP the amplitude of the NMDAR EPSC was unaltered, suggesting that GluN2B-NMDAR were replaced by GluN2A-NMDAR. In our study we only focused our attention on the dynamic change that occur during the first minutes so we cannot exclude the possibility that later on there is an increase contribution of GluN2A-NMDAR to synapses. To test whether such a late mechanism could actually play a role as crucial as the one of the activity-dependent GluN2B-NMDAR surface dynamic up-regulation, we performed a new series of experiments which demonstrate that GluN2A x-link does not prevent LTP (page 10). Thus, if GluN2A-NMDAR are laterally trafficking to synapses this mechanism does not play a crucial role for the induction of LTP. The new series of experiment has now been included in the revised manuscript.

While the control experiments in Fig3 that prove that the x-linking process doesn't impair normal synaptic function are rigorous, they don't directly contribute to the impact of the paper, as this technique has been previously described (these papers are adequately referenced in the manuscript). Could some of Figures 3 and 4 be combined (as well as the corresponding sections within the Results), with some panels provided as supplementary figures.

Response: We conceptually agree with that remark, although this work is the first demonstration that NMDAR can be efficiently immobilized without producing side-effects on their channel properties. Our previous works focused on AMPA receptors specifically and it is obvious that while it did not affect AMPA receptor function, the NMDAR x-link procedure might have different consequences on NMDA receptor, especially considering that the antibodies used to this end did not target similar extracellular domains of the ones used for AMPA receptors. Thus we strongly believe that the demonstration that NMDAR can be immobilized without affecting receptor function is essential to the paper. For more clarity however, we fused Figures 3 and 4 (now Figure 3 in the revised manuscript) following the recommendation of the referee.

In figure 5 as GluN2A experiences no surface redistribution following chemLTP (Figure 1), it could be argued that GluN2A x-linking would be insufficient to block LTP. It would be nice to actually see this hypothesis tested.

Response: To directly address this question, we have now performed a new series of experiments which demonstrate that, as expected, GluN2A x-link does not prevent LTP, as the GluN1 or GluN2B x-link does. This new data set is now described in the Results section of the revised manuscript (page 10).

The data relating to the experiments performed using anti-NMDAR autoimmune antibodies from patients with cognitive dysfunctions don't add much to the story and seem a little preliminary. There is scant information regarding their provenance - is this pooled IgG from multiple patients? Also the mechanisms of how these antibodies act is unclear if the antibodies are mixed they may be doing several things.For example are they targeting different epitopes of NR subunits? What impact do these antibodies have on NR function. It's quite possible some of the patient IgG effects are not due to cross-linking per se. These data might be better suited to amore in depth study for a more clinically focused journal but if they are to be kept then their presentation might be improved. A quantification of the qd trajectories with patient and control IgG, represented in panel B, would be helpful and straightforward to create e.g. panel B of Figure 3. Are the lower panels of Figure 6 panel D comparable to panels C and D of Figure 5. I'm unsure why a.u. was employed as the scale *of measurement in the lower left graph of Figure 6 panel D, when the corresponding graph in Figure 5C uses %. Also, with particular reference to Control IgG, the numbers in Figure 6 differ quite dramatically with the numbers in Figure 5C and 5D. In lower left panel of Figure 6D, the GluA1 syn. content almost doubles over basal, compared to a modest 10% increase observed in the same control experiment in Figure 5C. Likewise, a modest increase in potentiated synapses in the lower right panel of Figure 6D contrasts with an almosttrebling in the corresponding experiment presented in Figure 5D. These discrepancies should be addressed.*

Response: Although we do agree that we, and the whole community, are only in the premise of understanding how anti-NMDAR autoimmune antibodies act to induce cognitive dysfunctions, we believe that our data provide meaningful information and should be kept for several reasons: i) they show that “natural” and commercial antibodies produce similar consequences on NMDA receptors, i.e. to prevent their surface diffusion without affecting their channel function, and ii) they suggest that NMDAR x-link-induced deficits in LTP could occur in patients suffering anti-NMDA encephalitis, which could in part explain their memory and cognitive impairments. Although patients’ antibodies are polyclonal and may thus target several part of the extracellular part of the receptor, a recent study has identified a specific amino acid sequence that would be the epitope of the IgG (Gleichman et al., J. Neurosci., 2012). This point has been indicated into the manuscript (page 13).
Regarding their mechanism of action, we recently demonstrated that as commercial anti-NMDAR antibodies, these autoimmune autoantibodies prevent the surface diffusion of NMDA receptors without affecting their channel function (Mikasova et al., Brain, 2012), which we simply confirmed here (Figure 6B, C). This really emphasizes the fact that these autoantibodies essentially act through a x-linking of NMDA receptors. We better clarify the point that the effect of these patients’ autoantibodies on GluN-NMDAR surface dynamics (various subunit), NMDAR calcium permeability, NMDAR surface content, synaptic anchoring, and receptor interaction, have recently and thoroughly been described by our (e.g. Mikasova et al., Brain, 2012) and other labs. We understand that our initial presentation of the past and current work around these autoantibodies was not optimal, we thus improve the revised manuscript accordingly (page 13, Method section page 25, and supplementary methods).
Finally, there is indeed a discrepancy in the unit expression of GluA1 synaptic content between former Figure 5C-D (now Figure 4 C-D) and Figure 6D. In one hand (Figure 5) we pooled all clusters from different neurons and expressed the percent of variation to control, whereas, on the other hand (Figure 6), we average the values of the clusters for each neuron and expressed the average as intensity arbitrary unit. The rationale for this distinction comes from a former publication of the lab (Mikasova et al., Brain, 2012) and related unpublished observations that showed that autoimmune antibodies can have in the *in vitro* preparations the tendency to differentially impact neurons. This led to strong variations of the staining over network fields. To reduce this effect, which can biased cluster sampling by emphasizing few highly-targeted neurons, we thus compare the average of separated neurons. Although we believe it is the best way to proceed, it should be stated that when the whole cluster populations were compared it provided similar variation as the one expressed in Figure 4 (+11 ± 4% after $_{\text{chem}}$LTP of GluA1 synaptic content; before 1944 ± 51 a.u., after $_{\text{chem}}$LTP 2171 ± 71 a.u., n = 96 clusters; $p < 0.01$). To clarify this point and avoid confusion, we have now included these values in the text of the revised manuscript (page 13).

The results on CaMKII trafficking are potentially very interesting and could support an explanation for how NR2B Diff increase might underpin plasticity (in the absence of an increase in NR2A) but the data are mostly correlative and the underlying mechanism are not clear. Can the authors further address how an increase in NR2B Diff. alters CaMKII trafficking and then demonstrate that blocking this blocks LTP.

Response: This concern has now been addressed through a new series of single-particle tracking and FRAP experiments. As discussed above (point 2), we first found that in basal conditions, CAMKII regulates the surface dynamics of GluN2B-NMDAR through a phosphorylation-dependent mechanism since several CAMKII antagonists (KN62, KN93, AIP2) massively reduced the diffusion of GluN2B-NMDAR, both synaptically and extrasynaptically (Figure 7C). Interestingly, part of this regulation depends on the direct interaction between GluN2B and CaMKII since GluN2B-R1300Q/S1303D in which the C-terminal interaction site for CAMKII is prevented (Strack et al., J. Biol. Chem., 2000) also displays significantly reduced surface dynamics (Figure 7C). Moreover, this mutant is not displayed away from synapses as wild-type GluN2B-NMDAR are during activity-induced potentiation, indicating that the direct interaction between GluN2B-NMDAR and CAMKII is required for this plasticity-elicited surface redistribution to occur (Figure 7D, E). Finally, we performed additional FRAP experiments showing that expressing this GluN2B mutant prevents $_{\text{chem}}$LTP-elicited changes in CaMKII intracellular diffusion, suggesting that the direct interaction between GluN2B-NMDAR and CAMKII is also necessary for activity-induced changes in CAMKII intracellular dynamics to take place (Figure 8C). Therefore, we believe that trough their cytosolic interaction, activity-induced changes in the surface diffusion of GluN2B-NMDAR also affect CaMKII intracellular dynamics in a crucial manner during synaptic plasticity induction. These results are now included in the Results section of the revised manuscript (Figures 7, 8, and corresponding text over the manuscript).

I find some of the text introducing quantum dots towards the beginning of the results section to be superfluous, as qds have been employed to study neuronal receptor dynamics for about 10 years and are relatively well established in the field. The results section might benefit from a more succinct treatment of this topic, especially given the sparsity of detail in the Methods sections, where this material might be more appropriately provided.

Response: The introduction on Quantum Dots in the Results section has been simplified (page 6) and the Methods/Supplementary methods have been completed accordingly.

Page 14: "Because surface NMDAR also directly interact with PSD-95" requires adequate referencing.

Response: This sentence is now illustrated by an appropriate reference: “Because surface NMDAR also directly interact with PDZ-containing scaffold proteins such as PSD-95 (Bard et al., PNAS, 2010)…”(page 16).

Figure 1: For panel A, can a corresponding images of Homer fluorescence be provided.

Response: we have now included a Homer fluorescence image in the panel A of Figure 1.

Figure 2 Panel B & C: how representative are the GluN2A images of panel B, as they don't appear to correlate well with the results summarised in panel C? From panel B, the vast majority of receptors appear to be localised at the synapse, as opposed to the perisynpase, but the quantified data in panel C suggests the opposite is true (as the ratio of synaptic over perisynaptic is around 0.5).

Response: To answer directly the question, we are absolutely confident that the image of GluN2A/QD distribution in the synaptic area of Figure 2 (panel B) is representative, as representative can be such an image knowing that there is a large variability between synapses. First, we can invite the referee to glance through our former publications and images, which were obtained in close conditions (Bard and Groc, 2011; Bard et al., 2010; Groc et al., 2006, 2007). Second, it is right that we calculated a 0.5 synaptic/perisynaptic ratio (a value not that different from the one of GluN2B-NMDAR), indicating that half the GluN2A-NMDAR are in the PSD and half in the perisynaptic area. However the dispersion of GluN2A is really different from that of GluN2B since most of the perisynaptic GluN2A-NMDAR are concentrated in the surrounding of the PSD, whereas most GluN2B are widely dispersed. By eye, it may thus give the impression that the image is indeed not representative but a sizeable fraction of perisynaptic GluN2A are present at the border of the PSD. This point has now been mentioned in the revised manuscript (page 29).

Figure 2D: There could be some mismatch between the Figure and the time periods mentioned in the accompanying figure legend. It says the recording lasted 35mins (should it be 40mins?) and that fluorescence increased 35mins after chemLTP (should this be 30mins?).

Response: The recording indeed lasted 35 min and the representative image was taken at 35 min. This has been clarified in the legend of Figure 2D in the revised manuscript.

Figure 2E: A control that allows for photobleaching, drift, etc, could be added to this graph, much like the first panel of Figure 1B.

Response: Thanks for pointing this out. This has now been corrected accordingly and the control for photobleaching ("Buffer") has been added to Figure 2E in the revised manuscript.

2nd Editorial Decision 18 December 2013

Thank you for submitting your revised manuscript for consideration by the EMBO Journal. It has now been seen by the two original referees whose comments are enclosed. As you will see, both referees are satisfied with the amount of revisions and thus support publication given that you address minor concerns raised below.

Given the referees' positive recommendations, I would like to invite you to submit a revised version of the manuscript, addressing the comments of referee #1.
I would be grateful if you could also once more revise your manuscript text, more specifically I would like to suggest:
- add the error bar indication (s.e.m.?) to all figures (as also indicated by referee #1)
- remove "these" in second line of abstract
- replace "tackle" with "address" throughout the text
- please also add author contributions and conflict of interest statements

I would also be grateful at this stage if you were to provide original source data, particularly uncropped/-processed electrophoretic blots for figure 8 of your manuscript. This is in accord with our policy to make original results better accessible for the community and thus increase reliability of published data. We would welcome one PDF-file per figure for this information. These will be linked online as supplementary "Source Data" files.

We will also publish in the HTML version of the paper a synopsis to complement the abstract and to allow an easy access to the main findings. I would be grateful if you were to provide 3-5 one sentence bullet points that highlight your findings and that are non-redundant with your abstract.

Please let me congratulate you at this point to this nice study! Thank you for contributing to the EMBO Journal.

REFEREE COMMENTS

Referee #1:

The authors thoroughly addressed previous concerns. They strengthen their work indicating that cross-linking GluN1 and GluN2B with antibodies against their extracellular N-termini impairs cLTP in parallel to impeding lateral diffusion of GluN2B-containing NMDARs.

Remaining minor concerns:

1. It seems not all scale bars are defined.

2. p14, 6th line from bottom: "involved in the regulation the basal dynamics" should read "involved in the regulation of the basal dynamics."

3. p15, 6th line from top: "suggesting a prominent of role" should read "suggesting a prominent role."

4. p19, 10th line from bottom: I assume "long-term depression in anion flow - and Ca2+-independent manner" should read "long-term depression in cation flow - and Ca2+-independent manner" as I don't think there is evidence that LTD depends on anion (Cl-?) influx.

Referee #2:

The manuscript has been adequately revised by the authors and includes several new experiments that a number of concerns raised by the reviewers. This is an interesting manuscript with an extensive series of well performed experiments that significantly add to our understanding of the mechanisms regulating NMDA receptors at synapses and their role in plasticity.